Negative regulation of myofibroblast differentiation by phosphatase and tensin homologue deleted on chromosome ten

Eric S. White, Rachelle G. Atrasz, Biao Hu, Sem H. Phan, Vuk Stambolic, Tak W. Mak, Cory M. Hogaboam, Kevin R. Flaherty, Fernando J. Martinez, Christopher D. Kontos, and Galen B. Toews.

ONLINE DATA SUPPLEMENT

MATERIALS AND METHODS (882 words)

Cell Culture and Reagents: C57Bl/6 embryonic mouse fibroblasts (mEF-1) and NIH 3T3 murine fibroblasts were from ATCC (Rockville, MD). Embryonic mouse fibroblasts lacking both *pten* (*pten*$^{-/-}$) alleles were described previously (E1). The FAK/Src inhibitor 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and its inactive control 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) were from EMD Biosciences (San Diego, CA). The PI3K inhibitor LY294002 was from Cell Signaling Technologies (Beverly, MA). Porcine TGF-β_1 was from R&D (Minneapolis, MN). Bisperoxo(pyridine-2-carboxyl)oxovanadate (bpV(pic)) was from EMD Biosciences (San Diego, CA). Unless otherwise indicated, all other reagents were purchased from Sigma.

Antibodies: Antibodies to phospho-Smad2, total Smad2, and Smad7 were from Cell Signaling Technologies. αSMA antibody (clone 1A4) was from Dako Corporation (Carpinteria, CA). β-tubulin antibody was from Upstate (Waltham, MA). Anti-human PTEN (clone 6H2.1) was from Cascade Biosciences (Winchester, MA). Neutralizing antibody against TGF-$\beta_{1,\ 2,\ 3}$ was from R&D. Fluorochrome-conjugated secondary antibodies were from BD Pharmingen.

Intratracheal bleomycin administration: Animals were anesthetized with pentobarbital. The trachea was exposed and sterile bleomycin sulfate (0.05U per mouse) or sterile PBS alone (50 μl) was delivered via a 26-gauge needle inserted directly into the trachea. Animals were monitored closely to ensure full recovery. All animal experiments received prior written approval from the University of Michigan Committee on the Use and Care of Animals. Animals were housed prior to, and during the course of experimentation, in specific pathogen-free rooms with *ad libitum* access to food and water.

Collagen measurements: Total lung collagen measurements were determined 21 days after bleomycin instillation. Secreted collagen from cultured cells was determined at indicated time points. Total lung collagen and secreted collagen from cultured cells were determined using the Sircol collagen dye binding assay as previously described (E2). Briefly, cells were lysed in non-denaturing lysis buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM $MgCl_2$, 2 mM EGTA, and 10% glycerol) containing protease inhibitors and clarified by centrifugation. 100 µl of the supernatant was mixed with 1 ml of Sircol Dye reagent and rocked at room temperature for 30 minutes, followed by centrifugation. The remaining pellet was resuspended in Sircol Alkali reagent and vortexed. Each sample (100 µl) was plated in a 96-well plate in triplicate, and absorbance was measured at 560 nm. Results were extrapolated against known concentrations of collagen and reported as total collagen (µg/100 µl) ± SEM.

Adenovirus construction: Adenovirus containing cDNA constructs encoding wild-type PTEN (Ad-PTEN) or the empty virus control (Ad-EV) were prepared as previously described (E3). All adenovirus work was carried out in Biohazard Level 2+ safety hoods as approved by the University of Michigan Institutional Biosafety Committee.

Immunofluorescent staining: Cells were plated in individual wells of 8-well glass slides (BD Falcon) and allowed to adhere overnight. Cells were fixed with methanol and were permeabilized with 0.5% NP-40 in PBS for 15 minutes at room temperature. Following washing in PBS, cells were blocked with normal goat serum for 30 minutes at room temperature and incubated with anti-αSMA antibodies (1:1000) for 1 hour. Cells were washed and then incubated with FITC-conjugated goat anti-mouse secondary antibody for one hour in the dark. Cells were again washed, mounted with DAPI-containing

mounting media (Vector Labs, Burlingame, CA), and viewed under an epifluorescent microscope.

Immunohistochemistry: Immunohistochemistry was performed on 3-5 μm sections of formalin fixed, paraffin embedded surgical lung biopsy specimens obtained from patients undergoing surgical lung biopsy for diagnosis of idiopathic interstitial pneumonia. Prior written informed consent was obtained from all subjects in accordance with the University of Michigan Institutional Review Board. Sections were deparaffinized and rehydrated through graded alcohol and water. Antigen retrieval was carried out using Antigen Unmasking Solution (Vector Labs) in a Tendercook Pressure Cooker in a microwave following the manufacturer's instructions. Tissue sections were blocked with a 20% serum solution derived from the animal in which the secondary antibody was generated for 1 hour at room temperature in a humid chamber. Tissue sections were then incubated with primary antibody followed by biotinylated secondary antibody, and stained using the Vectastain ABC Universal kit (Vector Labs). Color development was achieved using 3,3'-diaminobenzidine and sections were counterstained with hematoxylin. For immunofluorescent staining, the method of Mason et al (E4) was followed.

Fibroblast proliferation assay: Fibroblasts ($5x10^3$/well) were plated in 96-well plates and allowed to adhere overnight. Cells were then serum-starved for 24 hours. Media were changed, with indicated reagents added, and cells cultured for 24 hours. ^{3}H-thymidine was added for the last 16 hours of culture and radioactive counts per minute (cpm) were assessed using a scintillation counter. Results were reported as the mean cpm ± SEM.

Semi-quantitative real-time polymerase chain reaction (RT-PCR): Semi-quantitative RT-PCR was performed using primers and probes for αSMA and PTEN previously described (E5, E6), and GAPDH was used to normalize results. Relative gene expression was calculated using the comparative cycle-threshold (C_T) method (E7).

Western blot analysis: Western blot analysis under reducing conditions was performed on equal concentrations of protein from whole-cell lysates and immunoprecipitated proteins as previously described (E6).

Statistical analyses: Statistical analyses were performed using GraphPad InStat 3.05 (San Diego, CA). Differences between groups were evaluated using Student's *t* test. For multiple comparisons, one-way ANOVA with Bonferroni's post-test analysis was utilized. Data were considered significant if $p<0.05$. Results were plotted using GraphPad Prism 3.02 (San Diego, CA). Densitometry of visualized bands on Western blot was performed using Image J software (version 1.31, NIH).

E1. Stambolic, V., A. Suzuki, J. L. de la Pompa, G. M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J. M. Penninger, D. P. Siderovski, and T. W. Mak. 1998. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 95(1):29-39.

E2. Kolodsick, J. E., G. B. Toews, C. Jakubzick, C. Hogaboam, T. A. Moore, A. McKenzie, C. A. Wilke, C. J. Chrisman, and B. B. Moore. 2004. Protection from fluorescein isothiocyanate-induced fibrosis in IL-13-deficient, but not IL-4-deficient, mice results from impaired collagen synthesis by fibroblasts. *J Immunol* 172(7):4068-76.

E3. Huang, J., and C. D. Kontos. 2002. Inhibition of vascular smooth muscle cell proliferation, migration, and survival by the tumor suppressor protein PTEN. *Arterioscler Thromb Vasc Biol* 22(5):745-51.

E4. Mason, D. Y., K. Micklem, and M. Jones. 2000. Double immunofluorescence labelling of routinely processed paraffin sections. *J Pathol* 191(4):452-61.

E5. Liu, T., S. M. Dhanasekaran, H. Jin, B. Hu, S. A. Tomlins, A. M. Chinnaiyan, and S. H. Phan. 2004. FIZZ1 stimulation of myofibroblast differentiation. *Am J Pathol* 164(4):1315-1326.

E6. White, E. S., V. J. Thannickal, S. L. Carskadon, E. G. Dickie, D. L. Livant, S. Markwart, G. B. Toews, and D. A. Arenberg. 2003. Integrin alpha4beta1 regulates migration across basement membranes by lung fibroblasts: a role for phosphatase and tensin homologue deleted on chromosome 10. *Am J Respir Crit Care Med* 168(4):436-42.

E7. Fink, L., W. Seeger, L. Ermert, J. Hanze, U. Stahl, F. Grimminger, W. Kummer, and R. M. Bohle. 1998. Real-time quantitative RT-PCR after laser-assisted cell picking. *Nat Med* 4(11):1329-33.